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Filing Date	2/27/2002
First Named Inventor	Helen K. Purss
Group Art Unit	
Examiner Name	
Total Number of Pages in This Submission: 3	Attorney Docket Number: 56104576-32

ENCLOSURES *(check all that apply)*

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Firm or Individual name	Frank M. Gasparo
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56104576-32

IN THE UNITED STATES PATENT AND TRADEMARK OFFICE

Applicants:	Helen K. Purss et al		
Serial No.:	10/085,658	Filed:	February 27, 2002
Examiner:	Not Yet Known	Group Art Unit:	Not Yet Known
For:	POLYMERIC MEMBRANES AND USES THEREOF		

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Frank M. Gasparo, Esq. (Reg. No. 44,700) — March 11, 2002 Date

Commissioner for Patents
Washington, D.C. 20231

SUBMISSION OF PRIORITY DOCUMENT

SIR:

Applicants hereby submit a certified copy of the priority document: Australian Provisional Application No. PR3407 filed on February 27, 2001 in the name of Gradipore Limited.

The Commissioner for Patents is hereby authorized to charge payment of all fees associated with this communication to Deposit Account No. 02-0393.

Respectfully submitted,

Date: March 11, 2002

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Enclosure

AUSTRALIA

Patent Office
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I, LEANNE MYNOTT, MANAGER EXAMINATION SUPPORT AND SALES hereby certify that annexed is a true copy of the Provisional specification in connection with Application No. PR 3407 for a patent by THE UNIVERSITY OF MELBOURNE filed on 27 February 2001.

I further certify that the above application is now proceeding in the name of GRADIPORE LIMITED pursuant to the provisions of Section 113 of the Patents Act 1990.

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Fourteenth day of February 2002

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MANAGER EXAMINATION SUPPORT
AND SALES

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LEANNE MYNOTT
MANAGER EXAMINATION SUPPORT
AND SALES

AUSTRALIA

Patents Act 1990

~~The University of Melbourne~~
Gradipore Limited

PROVISIONAL SPECIFICATION

Invention Title:

Polymeric membranes and uses thereof

The invention is described in the following statement:

Polymeric membranes and uses thereof

Field of the invention

The present invention generally relates to polymeric membranes. In particular, the present invention relates to gel membranes and their use in, but not limited to, electrophoretic techniques and the like, methods of making them and articles made and formed therefrom.

Background of the invention

The development of new polymeric membranes is an area of intense commercial interest because of their usefulness in many different applications. Membranes can be defined as selective barriers between two phases. Efficient separation is achieved by the differential rate of movement of molecules, and is dependent on the properties of the separation medium, for example, porosity, pore size distribution, thickness, hydrophilicity, membrane fouling, etc. Examples of the driving force for the movement of molecules across the membrane includes concentration differences, pressure differences and electric potential difference (eg electrophoresis-based systems).

A wide variety of different materials have been utilised for producing membranes. In general, microporous membranes can be divided into two main groups: those formed physically and those formed chemically. Physically formed membranes can be controllably formed by careful manipulation of the solubility of polymers in solution. These physically formed membranes are produced by either diffusion induced phase separation techniques (DIPS) or temperature induced phase separation (TIPS). Physically formed membranes are useful for many applications including water purification, dialysis and protein separation. However, the techniques for reliably producing physically formed membranes of controlled pore size distribution are often complicated, expensive and not easily reproduced in the laboratory.

Chemically produced membranes are made via a series of chemical reactions to form very thin three-dimensional polymeric networks. Because these thin polymeric networks generally lack mechanical strength they are often supported by a substrate that provides the membrane with the requisite mechanical strength. Conventionally, these polymer membranes have been formed by chain polymerization. Examples of such polymer membranes include those formed from acrylics, vinylics, methyl methacrylates/ethylene glycol dimethacrylate (EGDMA) and acrylamide (AAm) / *N,N'*-methylene-bis-acrylamide (Bis) networks.

The present invention is particularly concerned with, but not limited to, membranes that are suitable for use in electrophoretic techniques. The Gradiflow technique is a particularly useful example of fixed boundary preparative electrophoresis method (US Patent No.'s US5650055 and US5039386 and WO0013776. This technique utilizes a semi-permeable membrane (hereinafter referred to as a "separation" membrane) to separate two streams (referred to as the "upstream" and "downstream") of macromolecular (eg proteins, DNA, RNA, etc) containing liquids. When an electric potential is applied across the membrane, charged species will move towards the electrodes. If the charged species are positively charged, they will move towards the negative electrode (cathode), conversely, negatively charged species would move towards the positive electrode (anode). Careful selection of the properties of the separation membrane (eg pore size distribution) will facilitate the separation of the desired charged macromolecules. Cooling of the solutions is accomplished by circulation of chilled buffer solutions that are separated by two further membranes, hereafter referred to as restriction membranes, and are situated between the electrodes and the separation membranes. The restriction membranes allow the passage of ions but not macromolecules. The arrangement of the membranes is illustrated by Figure 1.

Depending on the choice of separation, restriction and buffer characteristics, the Gradiflow technique can be used in at least four different modes: 1) Charged based separation, (2) size based separation, (3) concentration and (4) dialysis.

The prior literature concerning the Gradiflow method utilizes a thin polyacrylamide (PAAm) hydrogel membrane with a defined pore size (D. B. Rylatt, M. Napoli, D. Ogle, A. Gilbert, S. Lim, and C. H. Nair, *J. Chromatog., A*, 865, 145-153, 1999). The membrane is produced via the free radical co-polymerization of a monomer such as acrylamide (AAm) and a crosslinker such as *N,N'*-methylene-bis-acrylamide (Bis). These hydrogels are generally strong, flexible, chemically inert, bio-compatible and can be made with relatively controlled pore structure for most applications.

Recent work has facilitated advances into producing polymeric networks that satisfy the needs of further applications. One approach has focused on altering the nature of the monomers used, including changing the crosslinker, for example in the case of PAAm gels, substitution of Bis for another monomer can lead to a different network structure.(M. G. Harrington and T. E. Zewert, *Electrophoresis*, 15, 195-199, 1994; G. Y. N. Chan, P. A. Kambouris, M. G. Looney, G. G. Qiao, and D. H. Solomon, *Polymer*, 41, 27-34, 2000; G. Patras, G. G. Qiao, and D. Solomon, H., *Electrophoresis*, 21, 3843-3850, 2000) However, due to the free radical nature of the polymerization, careful

control of the reaction conditions is important. Failure to do so can lead to charged groups within the network and also reduced stability.

Generally, the pore size range of commercially available membranes is somewhat limited. For example, large pores suitable for DNA and RNA separations are not routinely available. Some of the unsolved problems remaining with conventional electrophoresis membranes include producing membranes with no or very few charged groups, the ability to control pore size over a wide range of pore sizes and the development of stable gels over a wide pH range.

It would therefore be beneficial to develop hydrophilic membranes having properties such as controllable pore sizes, good processability, reproducability, high resistance to degradation, bio-stability and bio-compatibility, and preferably, without one or more disadvantages of present systems.

Detailed description of the invention

The present invention provides a system of forming polymeric matrices by crosslinking a pre-polymer using any suitable chemistry. Unlike conventional free radical systems, where the crosslinked membrane is formed entirely by chain growth, the present invention involves the use of a pre-polymer that is crosslinked by a step-growth reaction with the crosslinker. This step-growth type of approach to forming a polymeric network allows for greater control over the properties of the polymeric network. The term "step-growth" (condensation growth) denotes the build-up of a polymer by gradual or stepwise growth with time. A consequence of these individual step reactions is that the network can be built up in a controlled fashion. Generally high conversion is preferred (>95%) to obtain high MWT. A schematic representation of the network formation is shown in Figure 2, where the pre-polymer has a plurality of hydroxy groups and X represents the crosslinking agent.

Accordingly, in a first aspect, the present invention provides a polymer gel membrane, the membrane being formed from a pre-polymer having a plurality of crosslinkable moieties and the crosslinkable moieties being crosslinked with a polyfunctional crosslinking agent.

The membrane of the present invention is particularly suitable for use in electrophoretic separation techniques.

Accordingly, in a second aspect, the present invention provides an electrophoretic medium for use in an electrophoretic technique, the electrophoretic medium consisting of or comprising a polymer gel membrane formed from a pre-

polymer having a plurality of crosslinkable moieties, the crosslinkable moieties being crosslinked with a polyfunctional crosslinking agent.

Preferably the polymer gel membrane is a hydrogel.

The pre-polymer may be a formed from a homopolymer or a copolymer.

Preferably the pre-polymer molecule is devoid of charge, or has a very limited charge. Preferably the crosslinkable moieties of the pre-polymer are suitably arranged so that they can react with crosslinkers.

Preferably the pre-polymer is substantially hydrophilic and has good water solubility.

Preferably, the crosslinkable moieties of the pre-polymer are hydroxy groups.

Preferably, the molecular weight range for the pre-polymer is in the range of 10-200, 000. Most preferably, pre-polymer has a molecular weight in the range of about 20, 000 to 30, 000.

The pre-polymer may be a natural or synthetic polymer. The synthetic pre-polymer may be formed by chain growth polymerization and/or by condensation polymerization.

A schematic representation of the steps that may be used to produce a polymer gel membrane in accordance with the present invention is shown in Figure 3. Careful selection of the pre-polymer used contributes to the control of the gel network architecture after crosslinking.

It is preferred that the pre-polymer is synthetically produced as greater control over the nature of the polymer can be exerted. Synthetically produced polymers are often more chemically inert and can readily be made to exacting specifications, including molecular weight, degree of branching and charge groups present.

On the other hand, polymers from natural sources can contain subtle variations in their structures, manifesting as variations in molecular weight, charged groups, etc. Because they are extracted from biological sources such as plants, their absolute structures vary according to the environment, genetic make up of the source, etc. For example, agarose is a polysaccharide consisting of 1,3-β-D-galactopyranose and 1,4-linked-3,6-anhydro-α-L-galactose units. However, due to the presence of charged residues, such as sulphate, pyruvate and carboxyl groups, agarose gel networks exhibit a negative charge, and therefore often exhibit undesirable electroendosmotic properties when exposed to an electric field, and so are less preferred.

The term electroendomosis or electroendomostic property denotes the bulk fluid flow through membranes caused by the presence or acquisition of an electrical charge. The charged membrane will tend to respond to the application of an external electric field,

but because they are not free to move with respect to the electrolyte solution (buffer), there will be a movement of the electrolyte through the membrane. For example, a negatively charged membrane will cause solution to migrate towards the -ve electrode under the influence of a potential difference. There are techniques available to limit the amount of charged species present, but this dramatically increases the price of the polymer. Additionally, depending on the properties of the buffer solution, it is often possible for the membranes to develop a partial charge by the absorption of ions during the electrophoresis. We have this feature particularly useful in the present invention to control the flow of buffer through the membranes.

Examples of natural pre-polymers include, but are not limited to, starch, dextrans, cellulose derivatives, agarose, modified agaroses and other polysaccharides.

Examples of synthetic pre-polymers include, but are not limited to, poly(vinyl alcohol) (PVAl), partially esterified poly(vinyl alcohols), copolymers of poly(vinyl alcohols), polymers of hydroxyethylmethacrylate and hydroxyethylacrylate, and glycidylacrylate and glycidylmethacrylate and various copolymers thereof.

Preferably, the pre-polymer is a polyol. A particularly preferred example of a pre-polymer is poly(vinyl alcohol). PVAl may be prepared by the hydrolysis of poly(vinyl acetate) (PVAc), which is synthesized via the free radical chain polymerization of vinyl acetate. The level of hydrolysis is easily controlled, giving polymers with varying amounts of free hydroxyls. The molecular weight of the polymer can also be controlled during the polymerization of the vinyl acetate monomer.

The pre-polymer may be crosslinked by a variety of condensation chemistries to form extended polymeric matrices, for example, the crosslinking reaction may involve acetalization, etherification or esterification.

The crosslinking agent may be any reagent having at least 2 functional groups that are capable of undergoing reaction with the crosslinkable moieties of the pre-polymer to form covalent bonds. Preferably, the crosslinker itself is uncharged and be such as not to give rise to charged groups via side reactions. The products of the crosslinking reaction are also preferably relatively chemically stable. Preferably, any decomposition of the crosslinker will not lead to the development of charged groups within the polymeric matrix. The crosslinker is preferably relatively hydrophilic. The reactive groups in the crosslinker can be chemically equivalent or they may be of different chemical reactivity. Acid, base, or any other suitable catalyst known in the art may catalyze the crosslinking reaction. The crosslinking reaction is carried out under conditions such that the resultant crosslinked product is in the form of a hydrogel. Preferably the crosslinking reaction is performed under atmospheric pressure at a

temperature in the range of 10 to 60°C, more preferably 20 to 40°C. Preferably, the crosslinking reaction is carried out under atmospheric pressure and room temperature. Further control over the rate of crosslinking reactions can be exerted via adjustment of the concentration of acid catalyst added.

Examples of suitable multifunctional crosslinkers include, but are not limited to, dialdehydes, such as glutaraldehyde, preferably of controlled chain length; di-isocyanates, such as C_2-C_4-alkylene di-isocyanates, eg ethylene di-isocyante; diacids, such as maleic or oxalic; water soluble epoxides; diesters; diacid halides; free or etherified *N*-methylol ureas or *N*-Methylol melamines, such as *N,N*-dimethyolurea, N,N-dimethyolurea dimthyl ether or trimethyolmelamine dimethyl ether; dihalogen compounds, or epichlorhydrin, dianhydrides, dicarboxylic acids, citric acid, dicarboxylic,olefinin dialdehydes (eg propanedialdehyde), phthalaldehyde, 1,3-dichloroacetone and 1,3-dichloroisopropanol.

Preferably, the crosslinking agent is a dialdehyde. Examples of suitable dialdehydes include glutaraldehyde, 2-hydroxyhexanedial-1,6, malonic dialdehyde, succinic dialdehyde and hexanedial-1,6. Most preferably, the crosslinking agent is glutaraldehyde.

In a particularly preferred embodiment of the present invention, the polymeric membrane is formed from a poly(vinyl alcohol) as the polymer crosslinked with glutaraldehyde.

Accordingly, in a third aspect the present invention provides a polymeric gel membrane that is formed from a poly(vinyl alcohol) crosslinked with glutaraldehyde.

Preferably, the pre-polymer is crosslinked at levels of about 1 to 20%w/w crosslinker/polymer chain.

We have found that by controlling the ratio of pre-polymer to crosslinker, the network properties can be manipulated. The properties of the network depend on both the amount of crosslinking agent (eg glutaraldehyde) and on the molecular weight of the pre-polymer. Not being bound by theory, it is expected that there is a connection between the molecular weight of the polymer and the amount of crosslinker needed.

Contrary to the previous literature work on PVAl-glutaraldehyde gels it has been found that by controlling the ratio between PVAl and the crosslinker, glutaraldehyde, the properties of the network can be controlled, including mechanical strength, porosity, opacity, etc. By careful control and selection of the ratio of crosslinker to polymer, we have found that it is possible to produce networks with desirable pore sizes.

The lower the molecular weight of the pre-polymer, the more crosslinker required obtaining an equivalent network formation. Not being bound by theory, the

relationship between pre-polymer molecular weight and concentration of crosslinker is given by the Figure 4.

The preferred percentage weight range of the crosslinker in the polymeric membrane is between about 1% and 20%, more preferably between about 4 to 15 and most preferably, about 4.5-9.2%.

Preferably, the percentage of pre-polymer in the membrane is in the range of about 5-40%, more preferably 5 to 20.

The polymer gel membrane of the present invention may be self-supporting or it may be supported by a substrate(s).

In yet a fourth aspect, the present invention provides a membrane of the first aspect supported by a substrate.

The substrate is preferably formed from a material that is relatively inert, has good wet strength and does not bind to the substance undergoing separation (eg proteins). The substrate may be woven or non-woven material or a textile. The substrate may be in the form of a sheet, web, or any other appropriate form. The polymer gel layer may form on a surface of the substrate or the substrate may be within the gel.

The substrate may be formed from any material that is conventionally used as a membrane support. Non-limiting examples of suitable materials for use as substrates include, but are not limited to polyvinyl alcohol, polyethyleneteraphthalate (PET), nylon and fibreglass, cellulose, cellulose derivatives, or any other suitable substrates.

An example of a suitable substrate is heat bonded PET. Because of its hydrophobic nature, PET requires some pre-treatment to enable better wetting of the surface by the aqueous monomer solution. The surface may be pre-treated with a non-ionic surfactant, which renders the PET more hydrophilic while not introducing any charged groups into the system. It is, however, preferred that no pre-treatment of the substrate is necessary.

Preferably the substrate is hydrophilic nature in the case of aqueous solvent systems. An example of a hydrophilic substrate material is polyvinyl alcohol paper. Polyvinyl alcohol paper is strong white paper that has been found to be a suitable substrate. It is available in several different weights and thicknesses and may be used as the substrate without pre-treatment. An example of a suitable substrate is Papylon, the trade name for the PVAl paper (Sansho Corporation, The 2nd Kitahama Building 1-29, Kitaham-Higashi, Chuoh-Ku, Osaka, Japan, Ph: 06 6941 7895). Papylon has both excellent wet and dry strengths and has a very regular flat structure.

We have surprisingly found that in the case of the crosslinkable moieties being hydroxyl groups, hydroxyl coordinating buffers can be used to further decrease the pore size of the formed polymeric gel membrane, thus providing further flexibility in achieving a desired pore size for the membrane. Preferably, the coordinating agent is borate. Preferably the borate is in the form of a buffer.

Surprisingly another useful feature has been discovered resulting from the addition of the charged coordinating agent. Without wishing to be bound by theory, it is postulated that borate reacts with water to form an anionic borate ion with a negative charge. This is known to interact with 1,2- 1,3- and 1,4- diols to form negatively charged complexes. The complex formed between borate ions and PVAl induces an overall negative charge on the membrane surface, resulting in a net flux of buffer ions from the down stream to the upstream. The coordinating agent may be in the form of a buffer. The pH of the buffer may be selected to be within a particular range. The polymer-buffer interaction may be used to alter electroendosmotic flow. eg borate buffers of different concentrations between pH 7 and 9, to concentrate biomacromolecules such as DNA, RNA and proteins.Therefore the buffer may be used to control electrophoretic transfer or the rate of endosmosis.

Accordingly, in a fifth aspect, the present invention provides a polymeric membrane in accordance with the present invention that has been treated with one or more agent(s) that coordinates with the crosslinkable moiety.

Further control over the electroendomostic flow can be exerted in combination with the coordinating agent by the addition of a hydrogen bond breaker. The term "hydrogen bond breaker" is used herein in its broadest sense to denote any species that is capable of altering, modifying, controlling and or improving the hydrogen bonding characteristics of the pre-polymer component of the hydrogel. Without wishing to be bound by theory, it has been postulated that the addition of a hydrogen bond breaker disrupts the existing inter- and intramolecular hydrogen bonding of the hydroxyl groups of the pre-polymer component of the monomer. This allows for enhanced interaction between the hydroxyls and the charged complexing agent, such as the borate ion. The hydrogen bond breaker is preferably chosen from urea, formamide, melamine, guanidine, potassium acetate or derivatives thereof. Other hydrogen bond breakers will be known to those skilled in the art. A particularly preferred example of a hydrogen bond breaker is urea.

In a sixth aspect, the present invention provides a method of forming a polymer gel membrane in accordance with the present invention, the method comprising:

providing a pre-polymer having a plurality of crosslinkable moieties; and

contacting the pre-polymer with at least one polyfunctional crosslinking agent, under conditions to form said polymer gel.

In a seventh aspect, the present invention provides a method of separating molecules including:

providing a crosslinked polymer gel membrane in accordance with the present invention; providing a sample containing the molecules to be separated, and subjecting the gel and sample to a separation technique.

The method of the seventh aspect may be used for separating molecules, especially charged species, or species capable of bearing a charge such as bio-molecule. The bio-molecules may be proteins, DNA or RNA.

The separation technique may be an electrophoretic technique. This technique may be that described as the "Gradiflow" technique. This technique allows for the separation of molecules on the basis of size or charge under native conditions. The electrophoretic technique may be that disclosed in US 5,650,055, the entire disclosure of which is incorporated herein by reference.

The separation technique may include the use of borate in solution to concentrate protein samples electrophoretically to control protein transfer when using, for example, a 3-membrane arrangement involving 3 membranes in accordance with the invention located between restriction membranes.

Increasing the concentration of polymer in the membrane may reduce the rate of electroendosmosis. Even in combination with complexing buffers, higher concentrations of polymer leads to marked reductions in bulk flow of buffer. Not being bound by theory, it is believed that the higher concentrations of polymer (for example PVAl), can lead to larger crystalline demains, which interfere with the association of the buffers on the membrane, thus reducing the observed endosmosis effects.

As shown in Table 1, the various effects of buffer choice and polymer concentration are presented.

Table 1. Examples of membrane formulations and characteristics

Formulation[a]	Buffer[b]	Flow Rate ($mL\ min^{-1}$)	Estimated Pore Size (PS) (kDa)	Notes[c]
5 / 4.5	P	0.04	67 < PS	Transfer slow
5 / 4.5	TG	0.13	67 < PS < 340	
5 / 4.5	TG	0.10	67 < PS < 340	PVAl substrate
5 / 4.5	TG	1.50	67 < PS	TB added
5 / 6.8	TG	0.40	67 < PS	
5 / 4.5	TG	0.23		13-23 k PVAl
5 / 4.5	TG	0.20		13-23 k 89% H.
5 / 4.5	TG	0.12	67 < PS	89-98 k PVAl
5 / 4.5	TG	0.25	67 < PS	124-186 k PVAl
5 / 0.65	TG	0.20	67 < PS	124-186 k PVAl
5 / 0.96	TG	0.18		89-98 k PVAl
10 / 2.29	TG	0.13		
10 / 2.29	TG	0.23		13-23 k PVAl
10 / 2.29	TG	0.20		13-23 k PVAl 89% H.
20 / 9.2	TG	0.06	67 < PS < 340	
5 / 4.5	TB	1.40	PS < 340	
5 / 4.5	TB	0.31	PS < 340	NaCl added
5 / 4.5	TB	2.82		Urea added
5 / 4.5	TB	0.09		3 membrane
5 / 0.65	TB	1.80	67 < PS	124-186 k PVAl
10 / 2.29	TB	0.40		
10 / 2.29	TB	0.38		13-23 k PVAl
10 / 2.29	TB	0.40		13-23 k PVAl 89% H.
10 / 4.5	TB	0.35		
20 / 9.2	TB	0.04	no transfer	tight matrix

[a] % PVAl (w/v) / Glutaraldehyde (w/w) [b] TG = Tris-Glycine, P = Phosphate, TB = Tris-Borate
[c] 22 K MWt PVAl used unless otherwise stated

Control over rate of protein transfer by the addition of neutral salts may be used when using such a 3-membrane arrangement.

In an eighth aspect, the present invention provides a cartridge suitable for use in an electrophoretic device, the cartridge incorporating a polymeric gel in accordance with the present invention.

The cartridge may be any suitable cartridge known to those skilled in the art. The cartridge may be that described in US Patent No.s 5,650,055 and 5039386 and WO 0013776, the disclosures of which are incorporated herein in their entirety by cross reference.

In a ninth aspect, the present invention provides a device comprising at least one membrane in accordance with the present invention located between two restriction membranes.

The following embodiments are provided for the purpose of further illustrating the present invention but in no way are to be taken as limiting the present invention.

EMBODIMENTS OF THE INVENTION

Example 1

Pre-treatment of membrane substrate.

Unwoven poly(ethyleneterephthalate) (PET) sheets that served as a mechanical support were treated with aqueous solution of Teric BL8 (0.5% (v/v), Huntsman Corp. Australia) a non-ionic surfactant was used to improve surface wettability. The sheets were cut to 18 cm x 8 cm and placed on a glass sheet to cast the gel membranes.

Membrane Preparation

Example 2

Preparation of 5% PVAl Membrane crosslinked with glutaraldehyde at 4.5% (w/w).

A solution of PVAl (5% w/v, 10 mL MW 22 000, 97.5%-99.5% hydrolyzed) and 0.2 M HCl (0.333 µL 6.0 M solution) was prepared. To this, glutaraldehyde (91.5 µL 25% w/v in aqueous solution) was then added. The solution was poured across the treated PET support and allowed to stand at room temperature for 30 min. Membranes were then washed in excess distilled water to remove residual catalyst prior to use.

Example 3

Preparation of 5% PVAl Membrane crosslinked with glutaraldehyde at 6.8% (w/w).

A solution of PVAl (5% w/v, 10 mL MW 22 000, 97.5%-99.5% hydrolyzed) and 0.2 M HCl (0.333 µL 6.0 M solution) was prepared. To this glutaraldehyde (136.5 µL 25% w/v in aqueous solution) was then added. The solution was poured across the treated PET support and allowed to stand at room temperature for 30 min. Membranes were then washed in excess distilled water to remove residual catalyst prior to use.

Example 4

Preparation of 20% PVAl Membrane crosslinked with glutaraldehyde at 9.2% (w/w).

A solution of PVAl (20% w/v, 10 mL MW 22 000, 97.5%-99.5% hydrolyzed) and 0.05 M HCl (0.083 µL 6.0 M) was prepared. To this glutaraldehyde (732 µL 25% w/v in aqueous solution) was then added. The solution was poured across the treated PET support and allowed to stand at room temperature for 30 min. Membranes were then washed in excess distilled water to remove residual catalyst prior to use.

Example 5

Preparation of 5% PVAl Membrane crosslinked with glutaraldehyde at 1.08% (w/w).

A solution of PVAl (5% w/v, 10 mL, MW 89 000-98 000, 99+% hydrolysed) and 0.2 M HCl (0.333 µL 6.0 M solution) was prepared. To this glutaraldehyde (21.53 µL 25% w/v in aqueous solution) was then added. The solution was poured across the treated PET support and allowed to stand at room temperature for 30 min. Membranes were then washed in excess distilled water to remove residual catalyst prior to use.

Example 6

Preparation of 5% PVAl Membrane crosslinked with glutaraldehyde at 4.5% (w/w).

A solution of PVAl (5% w/v, 10 mL, MW 89 000-98 000, 99+% hydrolysed) and 0.2 M HCl (0.333 µL 6.0 M solution) was prepared. To this glutaraldehyde (91.5 µL 25% w/v in aqueous solution) was then added. The solution was poured across the treated PET support and allowed to stand at room temperature for 30 min. Membranes were then washed in excess distilled water to remove residual catalyst prior to use.

Example 7

Preparation of 5% PVAl Membrane crosslinked with glutaraldehyde at 0.65% (w/w).

A solution of PVAl (5% w/v, 10 mL, MW 124 000-186 000, 99+% hydrolysed) and 0.2 M HCl (0.333 µL 6.0 M solution) was prepared. To this glutaraldehyde (12.98 µL 25% w/v in aqueous solution) was then added. The solution was poured across the treated PET support and allowed to stand at room temperature for 30 min. Membranes were then washed in excess distilled water to remove residual catalyst prior to use.

Example 8

Preparation of 5% PVAl Membrane crosslinked with glutaraldehyde at 4.5% (w/w).

A solution of PVAl (5% w/v, 10 mL, MW 124 000-186 000, 99+% hydrolysed) and 0.2 M HCl (0.333 µL 6.0 M solution) was prepared. To this glutaraldehyde (91.5 µL 25% w/v in aqueous solution) was then added. The solution was poured across the treated PET support and allowed to stand at room temperature for 30 min. Membranes were then washed in excess distilled water to remove residual catalyst prior to use.

Example 9

Preparation of 20% PVAl Membrane crosslinked with glutaraldehyde at 9.2% (w/w) on PVAl paper.

A solution of PVAl (20% w/v, 10 mL, 22 000, 97.5%-99.5% hydrolysed) and 0.05 M HCl (0.083 µL 6.0 M solution) was prepared. To this glutaraldehyde (732 µL 25% w/v in aqueous solution) was then added. The solution was poured across an untreated PVAl paper support and allowed to stand at room temperature for 30 min. Membranes were then washed in excess distilled water to remove residual catalyst prior to use.

Example 10

Cartridge Format 1.

For each electroendosmosis and protein separation test performed, a separating cartridge was assembled as per Figure 1. This used PAAm restriction membranes to prevent protein transfer from the upstream and downstream to the cooling buffer. Each PVAl membrane was used as a separation membrane between the restriction membranes. This system was used unless otherwise stated.

Example 11

Cartridge Format 2.

An alternative membrane cartridge system was used to the above system in order to examine the effects on electroendosmosis and protein separation, Figure 5. This comprised the same system as described already together with a crosslinked PVAl membrane, (marked 6, Figure 5, placed adjacent to the restriction membranes.

Electrophoresis

Before any protein separation or electroendosmosis tests were performed, it was necessary to ensure that the membranes used did not leak. A series of leak tests were used to ensure this.

Leak Testing.

An initial leak test was required to check the integrity of the membrane. Leakage would mean that the results obtained from subsequent testing and experiments would not be valid. Proteins to be separated according to their charge or size may leak through any holes. The peristaltic pump was switched on and any volume changes were recorded in the upstream and downstream at 1-minute intervals for 15 minutes. No volume change indicated that there was no leakage in the separation PVAl membrane.

The cooling buffer pump was then switched on together with the peristaltic pump to test the restriction membranes for leakage. Similarly to the initial leak test, no volume changes to the upstream and downstream indicated that these were not leaking.

Electroendosmosis Testing

Various buffers were used in the Gradiflow™ unit to determine the electroendosmostic rates through the membranes with the different levels of crosslinked PVAl. Electroendosmosis manifests itself as a volume change in either the up or downstream reservoirs. Several common buffers, 40 mM Tris-Borate at pH 8.5, 40 mM Tris-Glycine at pH 9.0, and 40 mM Phosphate at pH 7.0 were used for these tests. With both of the pumps switched on, electroendosmostic testing was conducted under the influence of a power supply at 200 V, 500 mA for 20 minutes. Volume changes both to the upstream and downstream reservoirs were monitored. These readings were recorded at 1-minute intervals.

Example 12

Electroendosmosis of 5% (w/v) PVAl crosslinked membranes with glutaraldehyde at 4.5% (w/w) in 40 mM Tris-Glycine Buffer, pH 9.0.

Electroendosmosis testing using the Gradiflow™ unit showed a flow rate of 0.13 $mLmin^{-1}$ from the downstream to the upstream reservoir.

Example 13

Electroendosmosis of 20% (w/v) PVAl crosslinked membranes with glutaraldehyde at 9.2% (w/w) in 40 mM Tris-Glycine Buffer, pH 9.0.

Electroendosmosis testing using the Gradiflow™ unit showed a flow rate of 0.06 $mLmin^{-1}$ from the downstream to the upstream reservoir. There was also a marked increase in the conductivity of this solution.

Example 14

Electroendosmosis of 5% (w/v) PVAl crosslinked membranes with glutaraldehyde at 4.5% (w/w) in 40 mM Tris-Borate buffer, pH 8.5 with the addition of NaCl (40 mM).

Electroendosmosis testing using 40 mM Tris-Borate buffer with 40 mM NaCl displayed a flow rate of 0.31 mL min^{-1} from the upstream to the downstream reservoir. The addition of salt increased the conductivity of the buffer solution from 0.959 mS to 2.82 mS.

Example 15

Electroendosmosis of 5% (w/v) PVAl crosslinked membranes with glutaraldehyde at 4.5% (w/w) in 40 mM Tris-Glycine, pH 9.0 with the substitution of Tris-Borate buffer, pH 8.5.

To confirm the induced electroendosmosis of the borate ion on crosslinked PVAl membranes, the downstream Tris-Glycine buffer sample was replaced with Tris-Borate buffer. Electroendosmosis testing showed a flow rate of 1.5 mL min^{-1} through the exchange of glycine for borate.

Example 16

Electroendosmosis rate determination using the alternative "3-membrane" cartridge c ntaining 5% (w/v) PVAl crosslinked membranes with glutaraldehyde at 4.5% (w/w) in 40 mM Tris-B rate buffer, pH 8.5.

Electroendosmosis testing showed a flow rate of 0.09 $mLmin^{-1}$ from the downstream to the upstream reservoir. The alternative membrane arrangement showed that volume increase in the upstream was reduced. Similarly, the volume decrease from the downstream by electroendosmosis was compensated with buffer replacement from the cooling buffer reservoir.

Example 17

Electroendosmosis of 5% (w/v) PVAl crosslinked membranes with glutaraldehyde at 4.5% (w/w) on PVAl paper in 40 mM Tris-Glycine Buffer, pH 9.0.

Electroendosmosis testing using the Gradiflow™ unit showed a flow rate of 0.10 mL min^{-1} from the downstream to the upstream reservoir.

Example 18

Electroendosmosis of 5% (w/v) PVAl crosslinked membranes with glutaraldehyde at 4.5% (w/w) on PVAl paper in 40 mM Tris-Borate Buffer, pH 8.5.

Electroendosmosis testing using the Gradiflow™ unit showed flow rate of 1.2 mL min^{-1} from the downstream to the upstream reservoir.

Protein Separation and Gel Electrophoresis.

Protein separation was examined using the various membranes and the buffer systems above. Protein samples were used to conduct a protein transfer from the upstream to the downstream of the Gradiflow unit with suitable buffer. These were prepared using either Bovine Serum Albumin (BSA, 67 kDa) and Hen Egg Ovalbumin (Ovalb, 45 kDa) samples prepared in 10 mL of the buffer used for separation, or 10 mL of human serum cryo-precipitate from plasma, containing a mixture of proteins including Fibrinogen (340 kDa) a large glycoprotein and smaller proteins such as Human Serum Albumin (HSA, 67 kDa) and Immunoglobin (IgG) (between 47 and 56 kDa). The latter was diluted with 20 mL of the buffer used for separation. Protein solution was placed in the upstream whilst the downstream was filled with the tested buffer. 100 µL fractions were taken from the upstream and downstream reservoirs at 10 minute intervals from time zero up to sixty minutes.

All gel electrophoresis was performed using Gradipore Ltd. ng21-420 gradient polyacrylamide (PAAm) gels. Gel electrophoresis of each BSA/Ovalb protein mixture

was performed under native conditions using 80 mM Tris-Borate buffer, pH 8.5 at 200 V, 500 mA, for 90 min. For the cryo-precipitate, electrophoresis was performed under reducing denaturing conditions. 50 µL fractions were taken from the upstream and downstream reservoirs at 10 minute intervals. These samples were reduced with 10 µL dithiothreitol (DTT) and electrophoresed under denaturing conditions with SDS Tris-Glycine buffer, pH 8.5 at 150 V, 500 mA, for 90 min. The protein bands were then stained with coomassie brilliant blue G-250 and washed with 10 % Acetic acid.

Example 19

Protein Separation using 5% (w/v) PVAl crosslinked membranes with glutaraldehyde at 6.8% (w/w) in 40 mM Tris-Glycine Buffer, pH 9.0.

BSA and Ovalb were tested for protein transfer across the membranes from the upstream to the downstream. Figure 6 shows complete protein transfer in less than 10 minutes across a 5% (w/v) PVAl membrane crosslinked with glutaraldehyde at 6.8% (w/w). Lanes 1-4 show protein fractions from the upstream at 10 minute intervals. Lanes 6-9 show protein fractions taken from the downstream at 10 minute intervals. Lane 10 contains a range of control molecular weight markers. The observed transfer suggests that the effective pore size of the membrane exceeds the 67 kDa size of BSA.

Example 20

Protein Separation using 20% (w/v) PVAl crosslinked membranes with glutaraldehyde at 9.2% (w/w) in 40 mM Tris-Glycine Buffer, pH 9.0.

Cryo-precipitate was tested for protein transfer across the membranes from the upstream to the downstream. Figure 7 shows successful transfer of some protein across a 20% (w/v) PVAl membrane crosslinked with glutaraldehyde at 9.2% (w/w). Lanes 1-4 show protein fractions from the upstream at 10 minute intervals. Lanes 6-9 show protein fractions taken from the downstream at 10 minute intervals. HSA was successfully transferred along with smaller proteins. This was not complete for HSA and the reduced fibrinogen subunits (47 and 56 kDa are visible, the remaining subunits are masked by the strong HSA protein band) have remained in the upstream and were not present in the downstream samples. This indicates that the molecular weight cut off of the membrane is below 340 kDa (and above 67 kDa) in size. Lane 10 contains a wide range control of molecular weight markers.

Example 21

Protein Separation using 20% (w/v) PVAl crosslinked membranes with glutaraldehyde at 4.5% (w/w) in 40 mM Tris-Borate buffer, pH 8.5.

Cryo-precipitate was tested for protein transfer across the membranes from the upstream to the downstream. Figure 8 shows successful protein transfer across a 20% (w/v) PVAl membrane crosslinked with glutaraldehyde at 4.5% (w/w). Lanes 1-4 show protein fractions from the upstream at 10 minute intervals. Lanes 6-9 show protein fractions taken from the downstream at 10 minute intervals. Lane 10 contains a wide range of control molecular weight markers. Examination of the electrophoresis gel in lane 9 shows successful transfer of all other proteins than Fibrinogen from the upstream to the downstream reservoir. Fibrinogen bands remained in the upstream system, also evident using the Tris-Glycine buffer system. The prevention of the 340 kDa protein from passing through the membrane indicated size exclusion by the membrane.

Example 22

Protein Separation using 20% (w/v) PVAl crosslinked membranes with glutaraldehyde at 9.2% (w/w) in 40 mM Tris-Borate buffer, pH 8.5.

Cryo-precipitate was tested for protein transfer across the membranes from the upstream to the downstream. Figure 9 shows no protein transfer at all across a 20% (w/v) PVAl membrane crosslinked with glutaraldehyde at 9.8% (w/w). Lanes 1-4 show protein fractions from the upstream at 10 minute intervals. Lanes 6-9 show protein fractions taken from the downstream at 10 minute intervals. Lane 10 contains a wide range of control molecular weight markers. The interaction between borate and PVAl combined with the increased concentration of PVAl and glutaraldehyde crosslinking tightens the effective pore size to such an extent that the proteins completely were restricted from transferring to the downstream. This induced size restriction that may facilitate concentration, desalting and buffer exchange processes for biomolecular samples.

Example 23

Protein Separation using 5% (w/v) PVAl crosslinked membranes with glutaraldehyde at 4.5% (w/w) in 40 mM Tris-Glycine, pH 9.0 with the substitution of Tris-Borate buffer, pH 8.5.

Protein samples containing Tris-Borate buffer were used substituted for Tris-Glycine buffer for this test. Tris-Glycine was used as the downstream buffer and the cooling buffer. BSA and Ovalb were tested for protein transfer across the membranes from the

upstream to the downstream. Figure 10 shows very slow protein transfer across a 5% (w/v) PVAl membrane crosslinked with glutaraldehyde at 4.5% (w/w). Lanes 1-4 show protein fractions from the upstream at 10 minute intervals. Lanes 6-9 show protein fractions taken from the downstream at 10 minute intervals. This gel demonstrates that borate used in the buffers significantly retards protein transfer through crosslinked PVAl membranes.

Example 24

Protein Separation using the alternative "3-membrane" cartridge containing 5% (w/v) PVAl crosslinked membranes with glutaraldehyde at 4.5% (w/w) in 40 mM Tris-Glycine buffer, pH 9.0.

Cryo-precipitate was tested for protein transfer across the membranes from the upstream to the downstream. Figure 11 shows successful transfer of some protein across a 5% (w/v) PVAl membrane crosslinked with glutaraldehyde at 4.5% (w/w). Lanes 1-4 show protein fractions from the upstream at 10 minute intervals. Lanes 6-9 show protein fractions taken from the downstream at 10 minute intervals. HSA was successfully transferred along with some smaller proteins. However, the larger proteins, including Fibrinogen were restricted from transfer from the upstream to the downstream.

Example 25

Scanning Electron Microscopy (SEM)

Gel structure morphology was examined using cryogenic SEM to prevent collapse of the gel network on drying. Gels 5 x 5 mm were mounted vertically on a SEM stub with a non-conductive glue and frozen at -198°C in liquid nitrogen. The top was fractured off and the gel then warmed to -85°C for 90 minutes whilst subliming water from the gel under reduced pressure. The sample was again cooled to -198°C and images of the fractured gel taken at various magnifications. Figure 12 shows pictures of PVAl gels crosslinked at different polymer concentrations. Both gel networks have uniform pore distributions and are clearly different to each other.

As will be seen from the discussion above, the present invention, in its preferred embodiments provides a surprising array of means for manipulating the pore size of the gels of the present invention. In particular, we found that when we used PVAl-glutaraldehyde gels, for example, in conjunction with the use of borate buffers, this resulted in a membrane with the following characteristics:

-control of pore size via amount of crosslinker added

-control of pore size by the stoichiometry of the borate
-additional control exerted by the addition of a hydrogen bond breaker, which has the effect of additional borate complexation, which allows for further control of the pore size

Any description of prior art documents herein is not an admission that the documents form part of the common general knowledge of the relevant art in Australia.

Throughout this specification the word "comprise", or variations such as "comprises" or "comprising", will be understood to imply the inclusion of a stated element, integer or step, or group of elements, integers or steps, but not the exclusion of any other element, integer or step, or group of elements, integers or steps.

It will be appreciated by those skilled in the art that numerous variations and/or modifications may be made to the present invention as shown in the specific embodiments without departing from the spirit and scope of the invention as broadly described. The present embodiments are, therefore, to be considered in all respects as illustrative and not restrictive.

Gradiflow is a trademark of Gradipore Limited.

Claims:

1. A polymer gel membrane, the membrane being formed from a pre-polymer having a plurality of crosslinkable moieties and the crosslinkable moieties being crosslinked with a polyfunctional crosslinking agent.

2. A polymer gel membrane according to claim 1 which is the form of an electrophoretic medium.

3. A polymer gel membrane according to claim 1 or claim 2, wherein the membrane is a hydrogel.

4. A polymer gel membrane according any one of the preceding claims, wherein the pre-polymer is formed from a homopolymer or a copolymer.

5. A polymer gel membrane according to claim 4, wherein the pre-polymer molecule is devoid of charge, or has a very limited charge.

6. A polymer gel membrane according to any one of the preceding claims, wherein the pre-polymer is substantially hydrophilic and has good water solubility.

7. A polymer gel membrane according to claim 6, wherein the crosslinkable moieties of the pre-polymer are hydroxy groups.

8. A polymer gel membrane according to any one of the preceding claims, wherein the molecular weight range for the pre-polymer is in the range of 10-200,000, preferably, 20,000 to 30,000.

9. A polymer gel membrane according to any one of the preceding claims, wherein the pre-polymer is a synthetic polymer formed by chain growth polymerization and/or by condensation polymerization.

10. A polymer gel membrane according to claim 9, wherein the synthetic pre-polymer is selected from the group consisting of poly(vinyl alcohol) (PVAl), partially esterified poly(vinyl alcohols), copolymers of poly(vinyl alcohols), polymers of

hydroxyethylmethacrylate and hydroxyethylacrylate, and glycidylacrylate and glycidylmethacrylate.

11. A polymer gel membrane according to claim 10, wherein the pre-polymer is poly(vinyl alcohol).

12. A polymer gel membrane according to any one of claims 1 to 8 wherein the pre-polymer is a natural polymer.

13. A polymer gel membrane according to claim 12, wherein the natural pre-polymer is selected from the group consisting of starch, dextrans, cellulose derivatives, agarose, modified agaroses and other polysaccharides.

14. A polymer gel membrane according to any one of the preceding claims, wherein the crosslinking agent is a reagent having at least 2 functional groups that are capable of undergoing reaction with the crosslinkable moieties of the pre-polymer to form covalent bonds.

15. A polymer gel membrane according to any one of the preceding claims, wherein the crosslinking agent is uncharged and is such as not to give rise to charged groups via side reactions.

16. A polymer gel membrane according to any one of the preceding claims, wherein the crosslinking agent is relatively hydrophilic.

17. A polymer gel membrane according to any one of the preceding claims, wherein the crosslinking agent is selected from the group consisting of dialdehydes, di-isocyanates, diacids, water soluble epoxides, diesters, diacid halides, free or etherified *N*-methylol ureas or *N*-Methylol melamines, dihalogen compounds, epichlorhydrin, dianhydrides, dicarboxylic acids, citric acid, olefinic dialdehydes, phthalaldehyde, 1,3-dichloroacetone and 1,3-dichloroisopropanol.

18. A polymer gel membrane according to claim 17, wherein the crosslinking agent is a dialdehyde.

19. A polymer gel membrane according to claim 18, wherein the crosslinking agent is selected from the group consisting of glutaraldehyde, 2-hydroxyhexanedial-1,6, malonic dialdehyde, succinic dialdehyde and hexanedial-1,6.

20. A polymer gel membrane according to claim 19, wherein the crosslinking agent is glutaraldehyde.

21. A polymer gel membrane according to any one of the preceding claims, wherein polymeric gel membrane is formed from a poly(vinyl alcohol) crosslinked with glutaraldehyde.

22. A polymer gel membrane according to any one of the preceding claim, wherein the pre-polymer is crosslinked at levels of about 1 to 20% w/w crosslinker/polymer chain.

23. A polymer gel membrane according to any one of the preceding claims wherein the percentage weight range of the crosslinker in the polymeric membrane is between about 1% and 20%, more preferably between about 4 to 15 and most preferably, about 4.5-9.2%.

24. A polymer gel membrane according to any one of the preceding claims, wherein the percentage of pre-polymer in the membrane is in the range of about 5-40%, more preferably 5 to 20.

25. A polymer gel membrane according to any one of the preceding claims, wherein the membrane is supported by a substrate.

26. A polymer gel membrane according to claim 25, wherein the substrate is woven, non-woven material or a textile.

27. A polymer gel membrane according to claim 25 or 26, wherein the substrate is in the form of a sheet or web.

28. A polymer gel membrane according to any one of claims 25 to 27, wherein the polymer gel membrane is a layer formed on a surface of the substrate or the substrate is within the polymer gel membrane.

29. A polymer gel membrane according to any one of claims 25 to 28, wherein the substrate is formed from a material selected from the group consisting of polyvinyl alcohol, polyethyleneteraphthalate (PET), nylon and fibreglass, cellulose and cellulose derivatives.

30. A polymer gel membrane according to claim 29, wherein the substrate is heat bonded PET, optionally pre-treated with a non-ionic surfactant.

31. A polymer gel membrane according to claim 25, wherein the substrate is hydrophilic nature.

32. A polymer gel membrane according to claim 31, wherein the substrate is polyvinyl alcohol paper.

33. A polymer gel membrane according to any one of the preceding claims, wherein the crosslinkable moieties are treated with a coordinating agent.

34. A polymer gel membrane according to claim 35 wherein the coordinating agent is in the form of a buffer.

35. A polymer gel membrane according to claim 33 or 34, wherein the coordinating agent is borate.

36. A method of forming a polymer gel membrane in accordance with any one of the preceding claims, the method comprising:

providing a pre-polymer having a plurality of crosslinkable moieties; and

contacting the pre-polymer with at least one polyfunctional crosslinking agent, under conditions to form said polymer gel.

37. A method of separating molecules including:

providing a crosslinked polymer gel membrane in accordance with any one of claims 1 to 35, providing a sample containing the molecules to be separated, and subjecting the gel and sample to a separation technique.

38. A method according to claim 37, wherein the molecule to be separated is a charged species, or a species capable of bearing a charge.

39. A method according to claim 38, wherein the molecule is a bio-molecule.

40. A method according to claim 39 wherein the bio-molecules is selected from the group consisting of proteins, DNA and RNA.

41. A method according to any one of claims 37 to 40, wherein the separation technique is an electrophoretic technique.

42. A method according to claim 41, wherein the technique allows for the separation of molecules on the basis of size and/or charge.

43. A method according to any one of claims 37 to 42, wherein sample is a protein and a borate in solution is used to concentrate the protein sample.

44. A cartridge suitable for use in an electrophoretic device, the cartridge incorporating a polymeric gel in accordance with any one of claims 1 to 35.

45. A device comprising at least one polymer gel membrane in accordance with any one of claims 1 to 35 between two restriction membranes.

Dated this twenty-seventh day of February 2001

Gradipore Limited
~~The University of Melbourne~~
Patent Attorneys for the Applicant:

F B RICE & CO

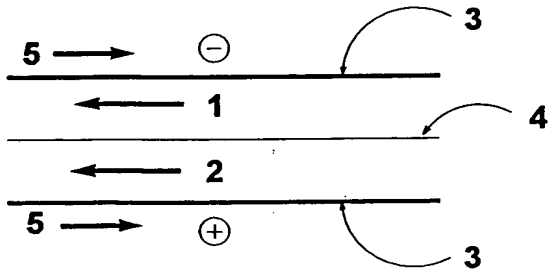

Figure 1. Schematic representation of membrane arrangement, where (1) upstream, (2) downstream, (3) restriction membrane, (4) separation membrane and (5) cooling buffer

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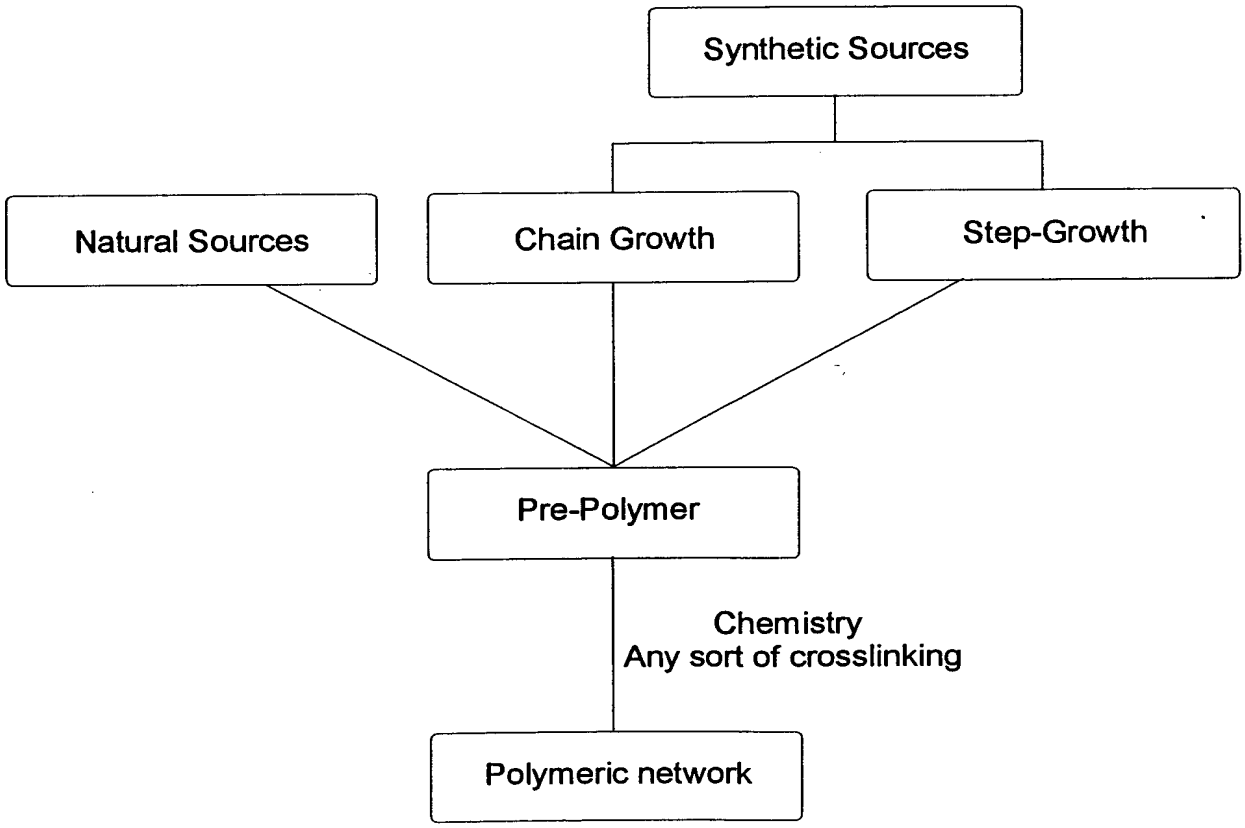

Figure 2 Sources of pre-polymers and formation of polymeric network

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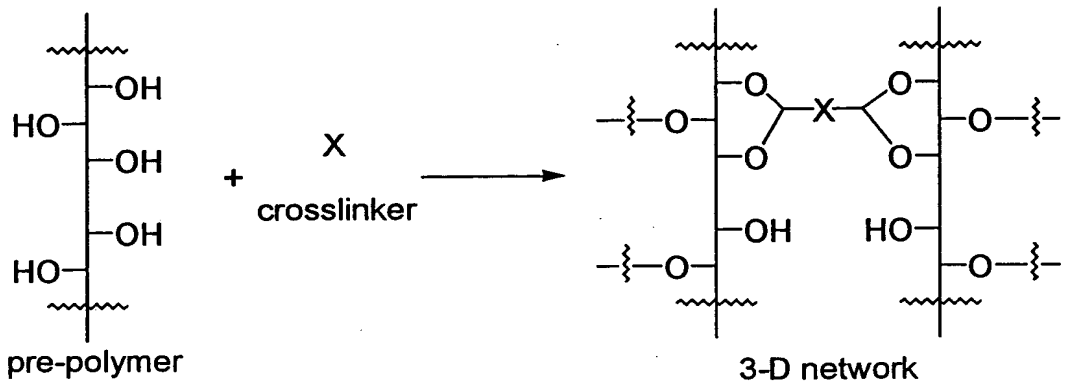


Figure 3. Linking hydroxy functional pre-polymers to form a polymeric 3D network

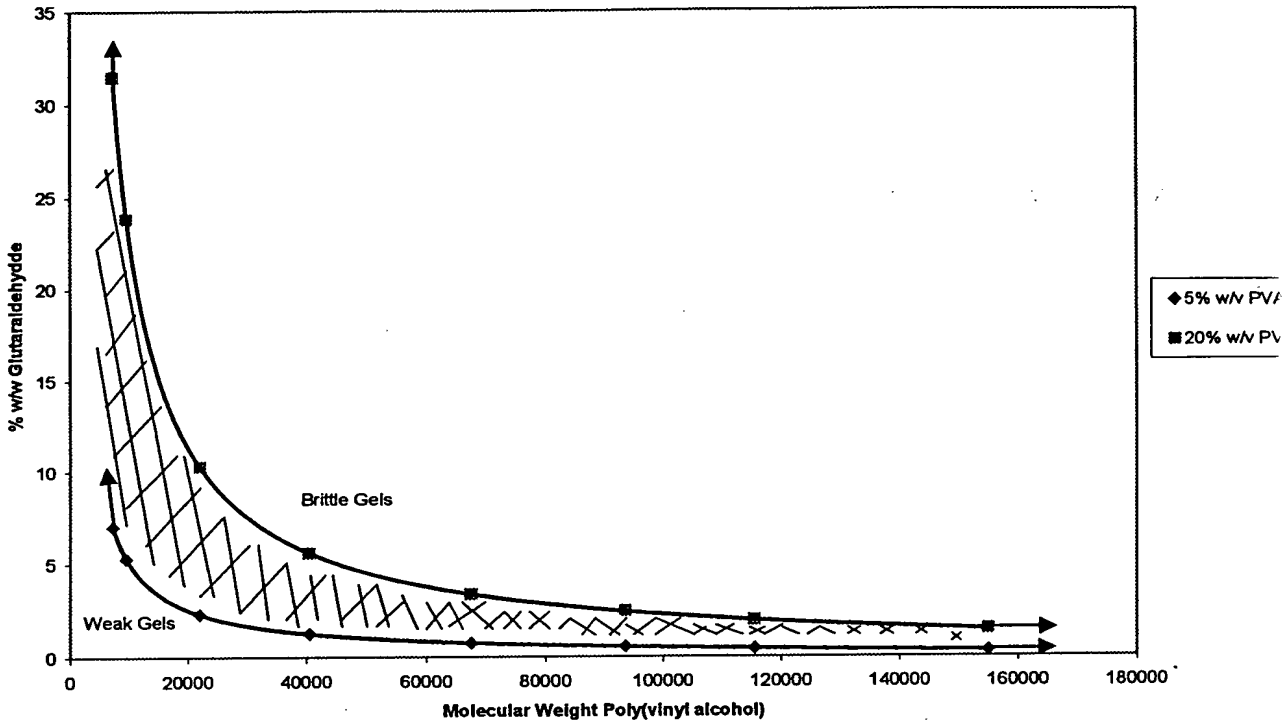


Figure 4. Relationship between molecular weight of polyol and % w/w crosslinker added

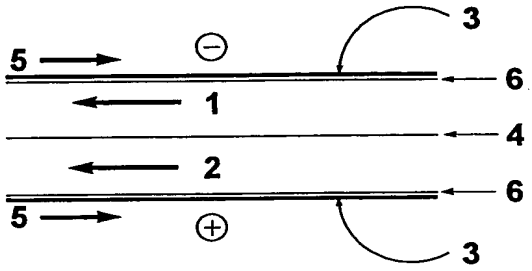


Figure 5 Schematic representation of 3-membrane arrangement, where **(1)** upstream, **(2)** downstream, **(3)** restriction membrane, **(4)** separation membrane, **(5)** cooling buffer and **(6)** crosslinked PVAl membrane

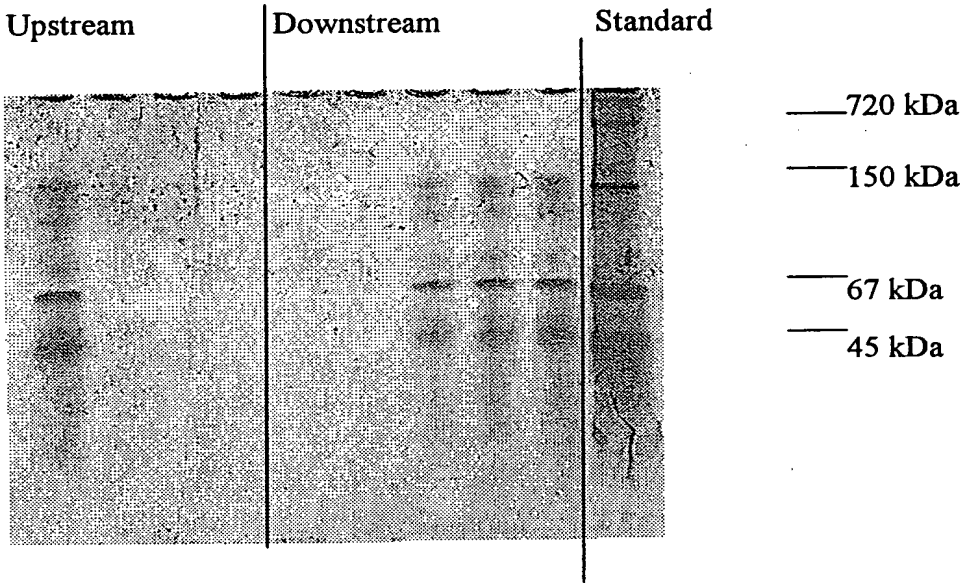


Figure 6

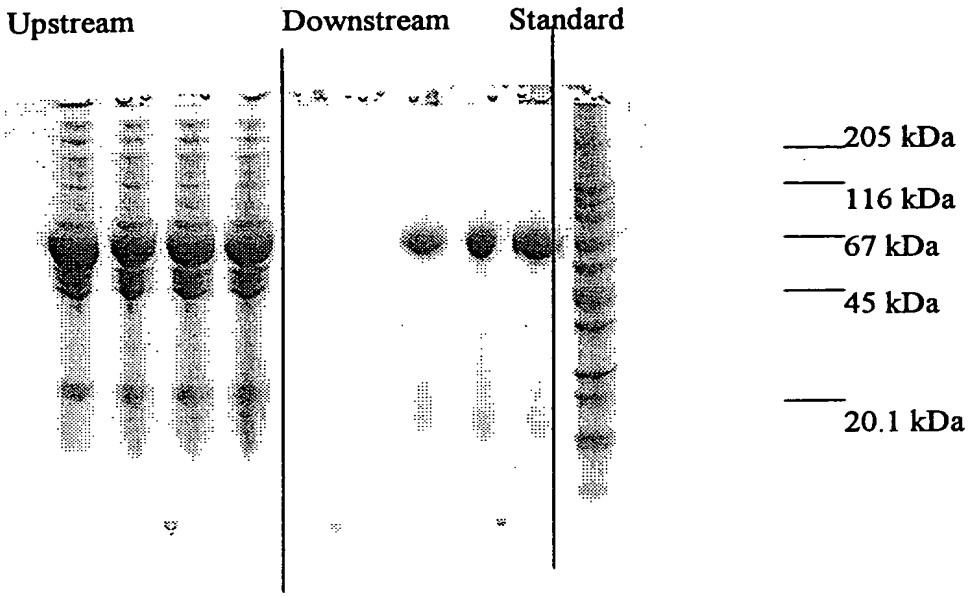


Figure 7

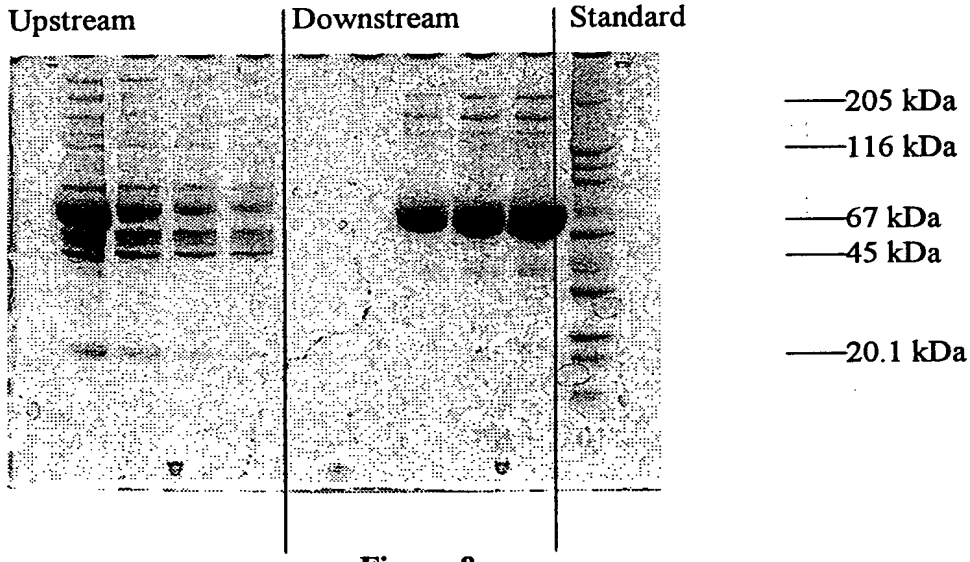


Figure 8

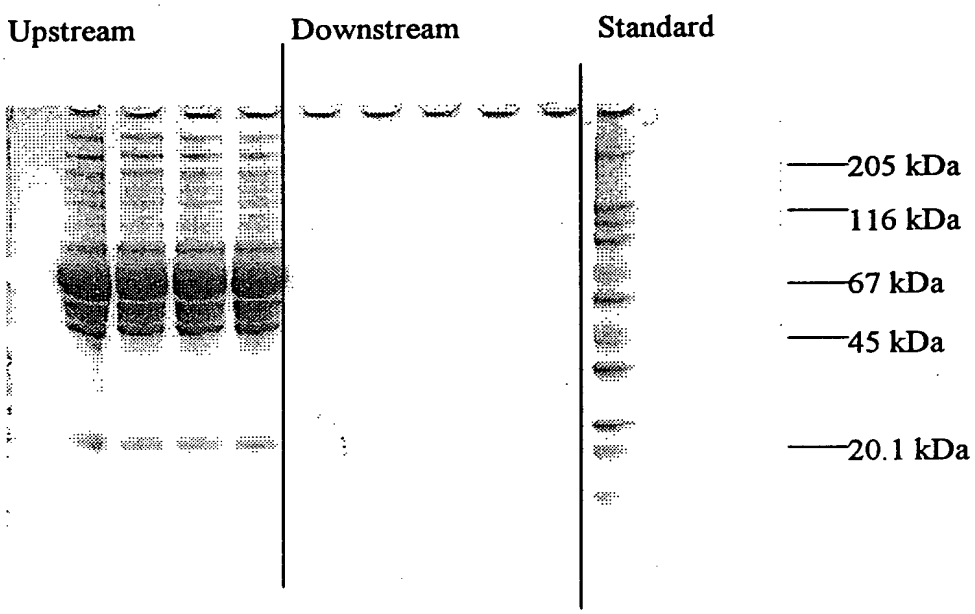


Figure 9

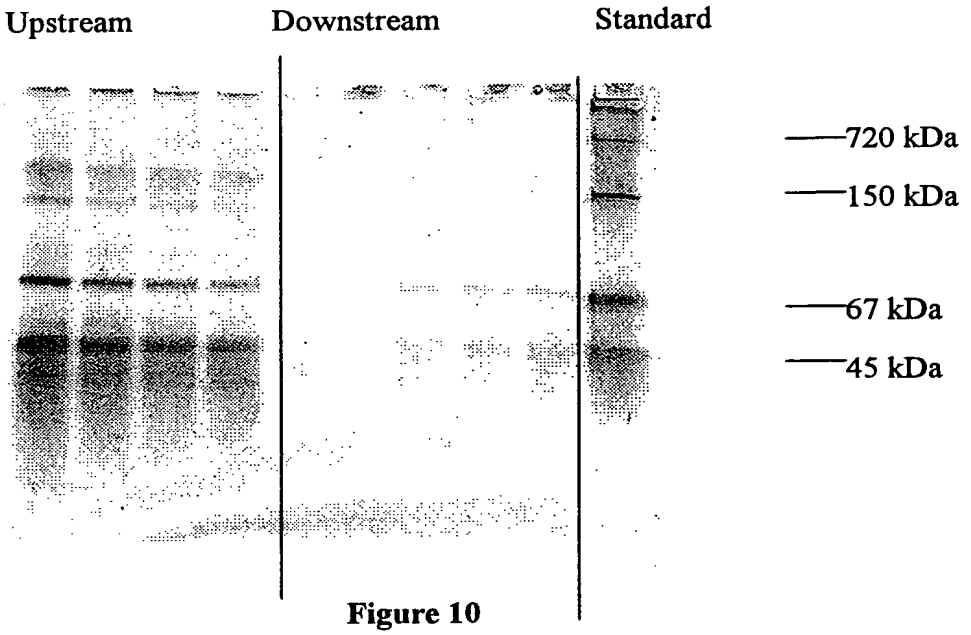


Figure 10

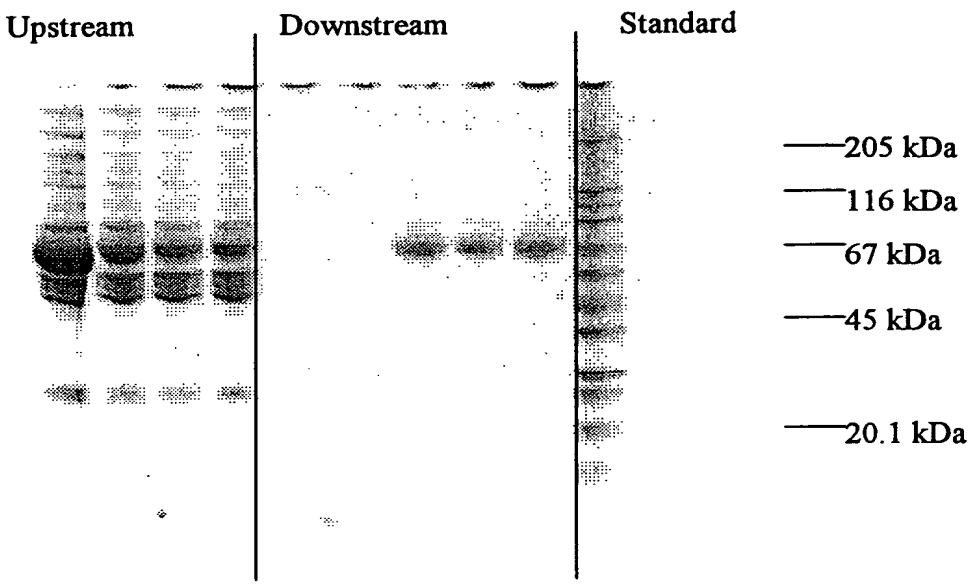
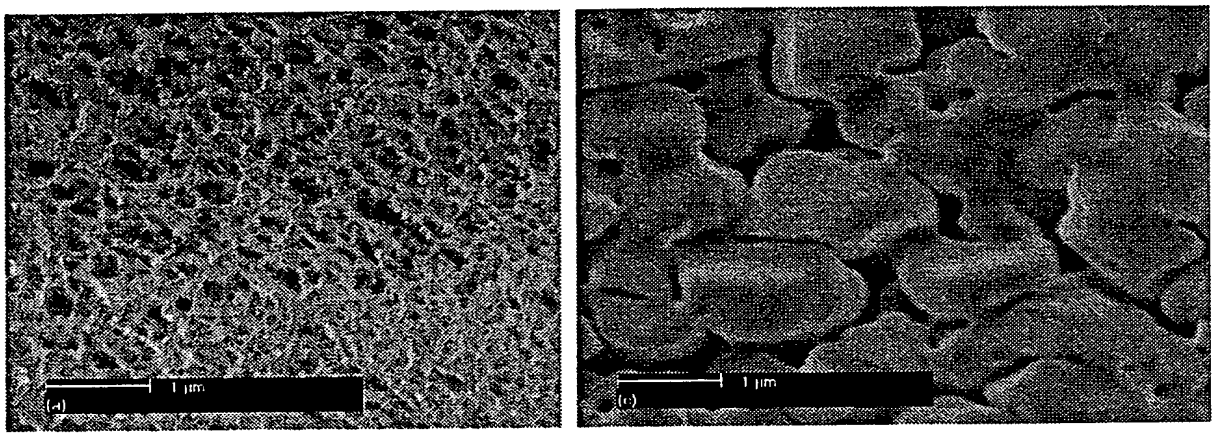

Upstream
Downstream
Standard
205 kDa
116 kDa
67 kDa
45 kDa
20.1 kDa

Figure 11



(1) (2)

Figure 12 SEM images, 15000 x magnification obtained for (1) 5% (w/v) PVAl crosslinked gel with glutaraldehyde at 4.5% (w/w) and (2) 20% (w/v) PVAl crosslinked gel with glutaraldehyde at 9.2% (w/w)